

# Investigations into Enzymatic Bioconversion to Form Rebaudioside A from Stevioside

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## Abstract

*Stevia rebaudiana* Bertoni is a perennial shrub from South America that produces steviol glycosides which are 200–300 times sweeter than sugar. Stevioside and rebaudioside A are the main sweetening components of its leaves. Steviol glycosides are diterpenoids whose biosynthetic pathways have four steps in common with gibberellic acid formation. The most important enzyme in the biosynthetic pathway expressed by the gene UGT76G1 is referred to as UDP-glycosyltransferase 76G1. It converts stevioside into rebaudioside A. The former has a bitter aftertaste and is a poorer sweetener but is most abundant. This enzyme can be produced in a next generation recombinant way by *Escherichia coli* and *Saccharomyces cerevisiae*. *Trichoderma longibrachiatum* produces the enzyme  $\beta$ -1,3-glucanase enzyme, which can perform a transglycosylation between stevioside to gain rebaudioside A. In our study, a full-factorial statistical experimental design that applies different glycosyl donors, temperatures, enzyme-to-substrate ratios and pH's as factors in order to achieve higher Reb A ratios in *S. rebaudiana* extracts after transglycosylation is reported. The presented statistical design was appropriate to indicate relevant and significant factors, providing a good basis for an upcoming experimental design of a real-world optimization.

## Keywords

*Stevia rebaudiana*, stevioside, rebaudioside A,  $\beta$ -1,3-glucanase, transglycosylation

## 1 Introduction

*Stevia rebaudiana* (Bertoni) (Fig. 1) is a member of the family Asteraceae. This plant originates from South America, especially Paraguay and Brazil in particular. *S. rebaudiana* is a small, herbaceous, perennial shrub of the family Compositae and native to certain regions of South America. The plant inhabited countries around the Pacific Rim, was cultivated domestically and applied in its raw leaf form. Finally it was processed commercially into sweeteners [1, 2]. The Guarani Indians of South America use *S. rebaudiana* to sweeten their tea. The sweetening components of these plants were described in 1931 [3]. The plant *S. rebaudiana* and its extracts have been used for an extensive period of time in China, Japan, Asia, South America and several countries in Europe. The leaves of *S. rebaudiana* and its highly refined extracts are used as low-calorie sweeteners in Korea, Japan and Brazil [4]. The extracts of *S. rebaudiana* have been approved by the Food and Drug Administration (FDA) and are generally recognized as safe (GRAS). This plant is gaining significance

and is expected to develop into a major source of high potency sweeteners for the developing natural food market. The sweetening of foods with extracts from *S. rebaudiana* extracts can increase the attractiveness of food by enhancing of flavors and odors. Sweeteners based on extracts from *S. rebaudiana* are used in beverages, table-top sweeteners, salty and processed food, cosmetics and various delicacies popular in Japan [5]. Stevioside, one of the steviol glycosides (SG's), has been reported to lower the postprandial blood glucose level of Type 2 diabetics and blood pressure of mildly hypertensive patients [6]. *S. rebaudiana* is used by diabetics as a form of diet therapy, and its extracts exhibit pharmacological effects such as anti-insulin resistance, the promotion of insulin secretion in addition to anti-hypertensive and anti-obesity properties [7]. Nowadays the utilization of alternative sweeteners has become a viable option to produce low-calorie or zero-calorie foods due to the growth of the healthy-food industry which intends to reduce the sucrose content of food products by totally or



Fig. 1 *Stevia rebaudiana*

partially replacing sucrose with using alternative sweeteners. The qualities of the food and beverages produced would support the use of *S. rebaudiana* sweetening components instead of sucrose or other sweeteners.

The major components of the plant *S. rebaudiana* are steviol, stevioside and rebaudioside A (Reb A).

The content of SG's is very important for optimal harvesting. The flowers and leaves are often valuable sources of SG.

The content of SG's found in *S. rebaudiana* varies from 4 to 20% of the dry weight of leaves depending on the cultivar and growing conditions. Stevioside is the main sweet component in the leaves of *S. rebaudiana* and tastes about 300 times sweeter than sucrose. Stevioside (13-[2-O $\beta$ -D-glucopyranosyl- $\alpha$ -glucopyranosyl) oxy] kaur-16-en-19-oic-acid $\beta$ -D-glucopyranosyl ester), is a glycoside with a glucosyl and a sophorosyl residue attached to the aglycone of steviol, which consists of a cyclopentanone-hydrophenanthrene skeleton [5].

Other minor SG components of lower concentration are steviolbioside, rebaudiosides A-F and dulcoside A [1].

The typical proportions of the major components of the leaves are stevioside (5–10% of the total dry weight of the leaves), Reb A (2–4%), Reb C (1–2%) and dulcoside A (0.4–0.7%) [5].

The highest total content of the SG's is located in the leaves. The contents of SG's in flowers and stems are 7-8- and 12-13-fold lower than in leaves, respectively. In the seeds the amount of the SG's are 2-2.5 times lower than in the flowers. The lowest content of SG's is found in the roots, in fact it is 40-fold lower than in the leaves [8].

## 2 The bioconversion of Stevioside to Rebaudioside A

The transformation of stevioside to Reb A is one of the most important bioconversions in this field of research area. Reb A has a more pleasant aftertaste and exhibits a higher sweetening effect than stevioside. The stevioside: Reb A ratio is a critical parameter in commercial products as well. Therefore, the aim of this study is to examine the transformation of stevioside to Reb A in primer aqueous *S. rebaudiana* extracts. To select a suitable enzyme, the following three candidates were compared. Firstly, UDP-glycosyltransferase UGT76G1 from *S. rebaudiana* catalyzes the conversion of stevioside to Reb A via an one-step glycosylation reaction. This enzyme could be produced by *E. coli* strain BL21(DE3) [9].

Secondly, hydrolases/transferases of *Gibberella fujikuroi* can convert stevioside into other relevant SG's, for example Reb A, Reb B and steviolmonoside [10].

Thirdly,  $\beta$ -1,3-glucanase from *Irpex lacteus* was applied by Singla et. al (2016) to develop a reaction pathway for an environmentally-friendly transglycosylation catalyzed by an enzyme. This enzyme could catalyze the bioconversion of stevioside to Reb A, and transfers the glucose from curdlan to stevioside (Fig. 2) at an appropriate bond position [11].

The aim of our work was to test different enzymes and glycosyl donors at different temperatures, different pH's and enzyme:substrate ratios (E/S) in order to increase the Reb A to stevioside ratio in *S. rebaudiana* extracts. To select relevant factors, a 2-level full factorial design, that provides a good basis for the experiment design of a real optimization, was generated.

## 3 Materials and methods

For the statistical evaluation, a two-level full factorial design, of which experimental settings and results are listed in Table 1 for each glycosyl donor, was implemented. There were 3 quantitative factors, namely pH, E/S ratio and T (°C). A qualitative factor referred to as a glycosyl donor, (i.e. water-soluble starch, sucrose, D-trehalose or curdlan) was also examined. Singla and Jaitak [11] used curdlan to conduct this bioconversion of steviol glycoside. 4 center points were made in addition to the  $2^3$  full factorial design, so in total  $(2^3 + 4) \cdot 4 = 48$  experiments were conducted.

The applied commercial enzyme preparatum (Sigma-Aldrich, Germany) contained mainly  $\beta$ -glucanase from *Trichoderma longibrachiatum* and exhibited mainly  $\beta$ -1 $\rightarrow$ 3 / 1 $\rightarrow$ 4-glucanase activities, but significant xylanase, cellulase,  $\beta$ -glucosidase,  $\beta$ -xylosidase,  $\alpha$ -L-arabinofuranosidase, amylase and protease activities were also present [12].

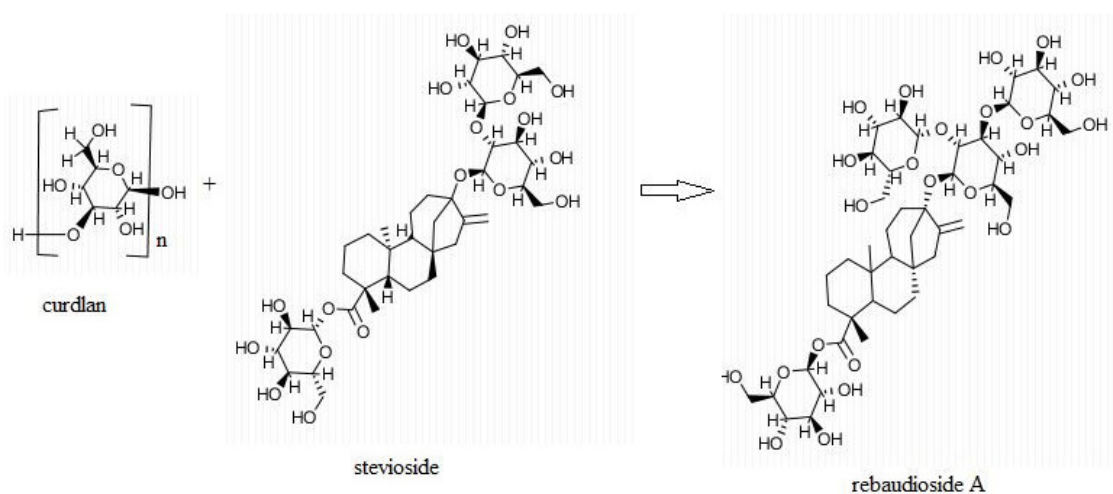


Fig. 2 Transglycosylation of stevioside to Reb A with  $\beta$ -1,3-glucanase

Table 1 Full factorial design and its results for all glycosyl donors

order	pH	E/S	T°C	DeltaRebA(%) water soluble starch	DeltaRebA(%) sucrose	DeltaRebA(%) D-trehalose	DeltaRebA(%) curdlan
1	5	3	25	25.275	13.333	-6.795	52.388
2	8	3	25	40.861	69.991	16.637	-86.765
3	5	9	25	-56.417	0.691	7.962	-41.474
4	8	9	25	0.284	18.891	-74.727	-39.866
5	5	3	45	28.480	-7.808	133.572	39.577
6	8	3	45	-7.387	-35.940	-40.225	-14.249
7	5	9	45	-22.369	-3.031	-86.796	-72.549
8	8	9	45	-42.717	27.103	23.738	1.664
9	6.5	6	35	4.223	-0.778	31.663	24.809
10	6.5	6	35	12.707	10.100	28.594	12.995
11	6.5	6	35	-35.566	26.819	-29.430	-37.574
12	6.5	6	35	3.380	16.129	-26.794	-16.616

The bioconversions took place in 15-ml Falcon conical centrifuge tubes in a temperature-controlled cabinet with a tube rotator equipment (Rotomix, Kutesz, Hungary) (Fig. 3).

The raw material was a pressurized hot-water extract (PHWE) of *S. rebaudiana* leaves. The *S. rebaudiana* extract was collected from the spontaneously dried plant material (2-3mm) produced by autoclaving (10 g plant: 200 ml distilled water) in a Tuttnauer 3870 ELV-type laboratory autoclave. After the PHWE (121°C, 20 mins) the extract was centrifuged (Hermle Z 200 A, 8 mins., 6000 rpm) to remove the solid components.

The *S. rebaudiana* extract and curdlan (from *Alcaligenes faecalis*, Sigma Aldrich, Germany) were mixed in a 1:2 ratio in a citrate buffer (0.1 M with different pH) and enzyme preparatum was added according to E/S ratio. The solution was mixed and incubated in a Rotomix overnight.

The reaction mixture was brought to room temperature and the enzyme deactivated by boiling it at 100°C for 5 mins.

Each sample was measured using high-performance liquid chromatography (HPLC) (ZORBAX Carbohydrate Analysis column) [13] to determine the increase in the peak area of Reb A compared to its initial peak area (deltaRebA(%)). The mobile phase consisted of a phosphate buffer and acetonitrile in 25:75 volume ratio. The eluent flow rate was 1 ml/min and a guard column was applied.

The equation of the calibration curve using HPLC was as follows where the peak area is denoted by y and the concentration of Reb A by x:  $y = 1000000 \cdot x + 116103$

The average and standard deviation of retention times were the following for the calibration of Reb A (Fig. 4): RT:  $4.565 \pm 0.044$  min



Fig. 3 Tube rolling equipment

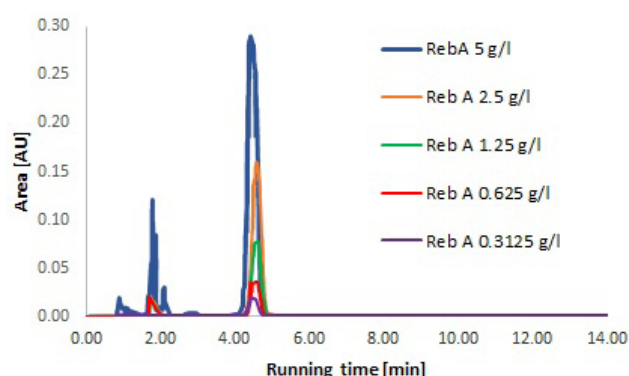


Fig. 4 HPLC overlay diagram: Reb A calibration

Minitab 17 software was used for the statistical evaluations.

#### 4 Results and discussion

The changes in the peak area of Reb A using HPLC were measured, which were expressed as  $\Delta\text{RebA}(\%)$  values. The  $\Delta\text{RebA}(\%)$  results showed how the chosen glycosyl donors (water-soluble starch, sucrose, D-trehalose and curdlan) effect the changes in Reb A concentration during the bioconversion.

For each glycosyl donor, the results were first analyzed from a statistical point of view. All residual plots were similar to Fig. 5 indicating that residuum, i.e. the differences between measured and fitted values, exhibit normal distribution, constant variance and are independent.

The first glycosyl donor to be tested was water-soluble starch. In Fig. 6A a Pareto chart is presented, which suggests that the effect of the E/S ratio is more significant than other effect and interactions.

The main effects of tested factors in the case of starch as a glycosyl donor are demonstrated in Fig. 6B, which suggests, that the application of a low-level E/S ratio will increase  $\Delta\text{RebA}(\%)$ , while the other factors exhibit

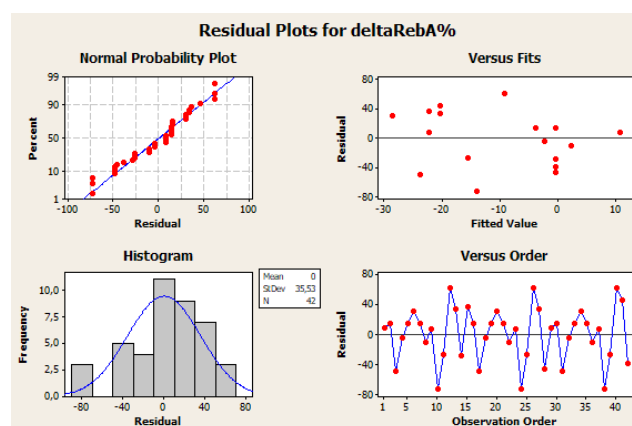


Fig. 5 Residual plots for  $\Delta\text{RebA}(\%)$

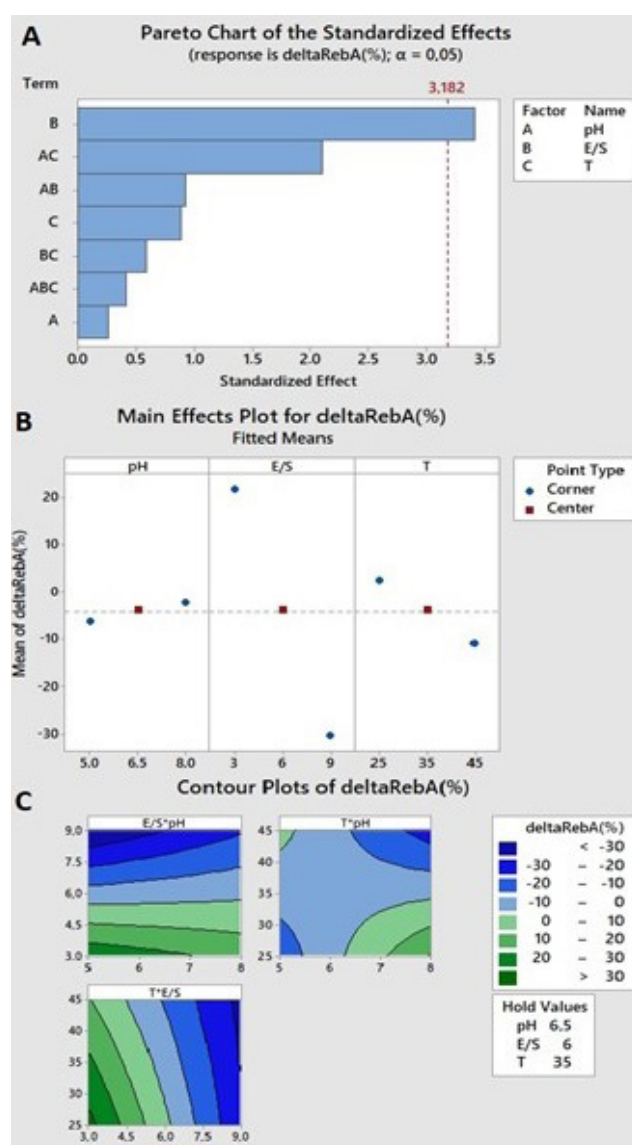


Fig. 6 Glycosyl donor: water soluble starch, A: Pareto Chart, B: Main Effects Plot, C: Contour Plot



minimal effects. In Fig. 6C the contour plots show how factors influence variation in terms of deltaRebA(%). Higher and better values are denoted by the dark green area, so low E/S ratios and temperatures are better for the formation of Reb A.

The second glycosyl donor to be examined was sucrose. According to the Pareto chart (Fig. 7A) the effect of temperature and interaction of E/S ratio and temperature were significant.

In Fig. 7B the main effects plot shows that the E/S ratio had no effect, but the low temperature and higher pH have a positive effect on the increase of deltaRebA(%).

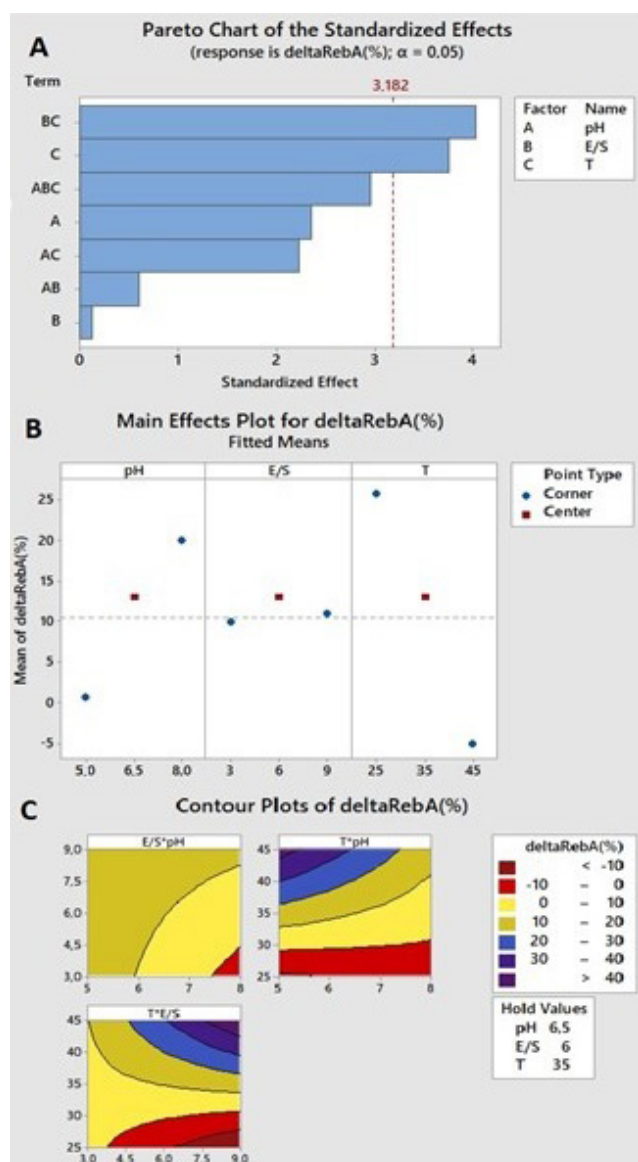


Fig. 7 Glycosyl donor: sucrose, A: Pareto Chart, B: Main Effects Plot, C: Contour Plot

In Fig. 7C the contour plots illustrate how the factors and their interactions affect deltaRebA(%). The dark purple area is preferred, i.e. high temperature, low pH and high E/S ratio cause an increase in deltaRebA(%).

The third examined glycosyl donor was D-trehalose. The Pareto chart in Fig. 8A shows that the interaction between the E/S ratio, pH and temperature has a significant effect on the increase in deltaRebA(%).

In Fig. 8B the main effects plot illustrates that the low levels of pH and E/S ratio in addition to the high temperature have some positive impression on the enhancement of deltaRebA(%).

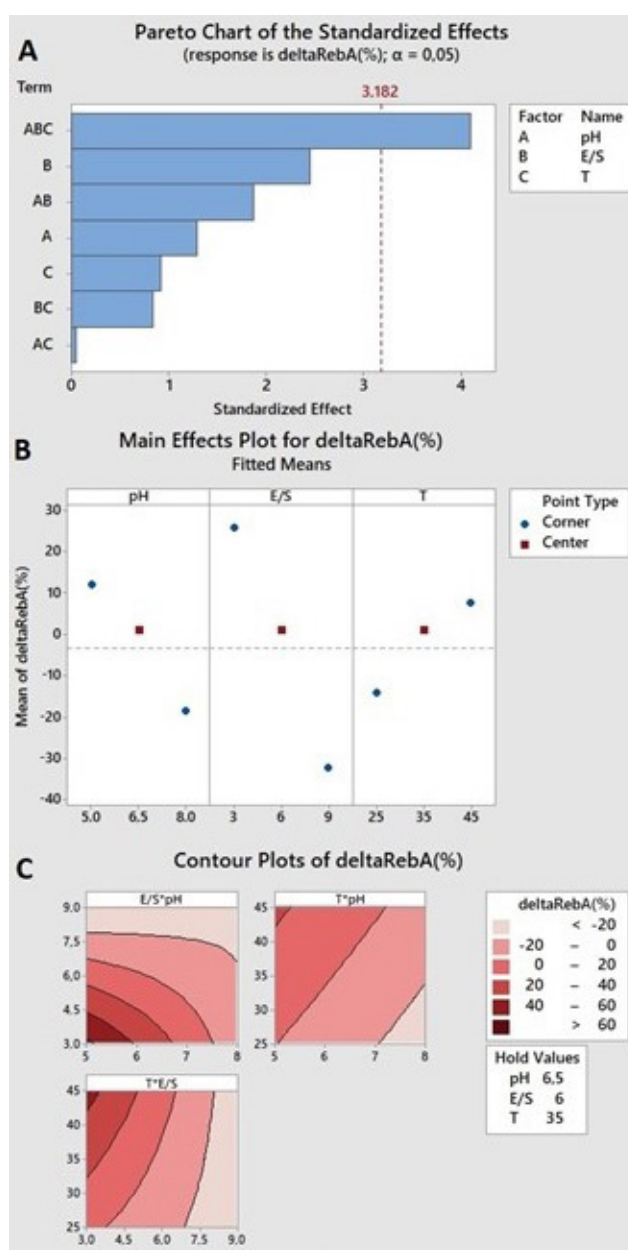


Fig. 8 Glycosyl donor: D-trehalose, A: Pareto Chart, B: Main Effects Plot, C: Contour Plot

In Fig. 8C the contour plots show how each factor and their interactions alters deltaRebA(%). The higher values of deltaRebA(%) are denoted by the dark red regions, therefore, the low pH, low E/S ratio and high temperature are preferable for the enhancement of deltaRebA(%).

In Fig. 9A (glycosyl donor: curdlan) the Pareto chart implies that the interaction between the pH and E/S ratio has a significant effect with regard to increasing deltaRebA(%) when compared to the other factors and interactions.

The main effects plot in Fig. 9B represents that the lower E/S ratio and pH in addition to the higher temperature have a positive effect in terms of the enhancement of deltaRebA(%).

In Fig. 9C the contour plots claim that the examined factors and their interactions effect deltaRebA(%). The dark blue area is the preferable, therefore, a low temperature, low pH and low E/S ratio results in a higher deltaRebA(%)

## 5 Conclusion

In this paper concerning the bioconversion of steviol glycoside, an attempt was made to identify an alternative glycosyl donor instead of curdlan, which has been frequently reported and applied, to effectively provide a glycosyl group for the enzymatic bioconversion of stevioside to Reb A. A two-level full factorial statistical design was implemented to screen for any significant effect of three factors, namely pH, E/S, and T°C.

For the purposes of identifying more detailed effects and correlations, new statistical experimental designs should be drawn up to improve the enzymatic bioconversion of stevioside to rebaudioside A.

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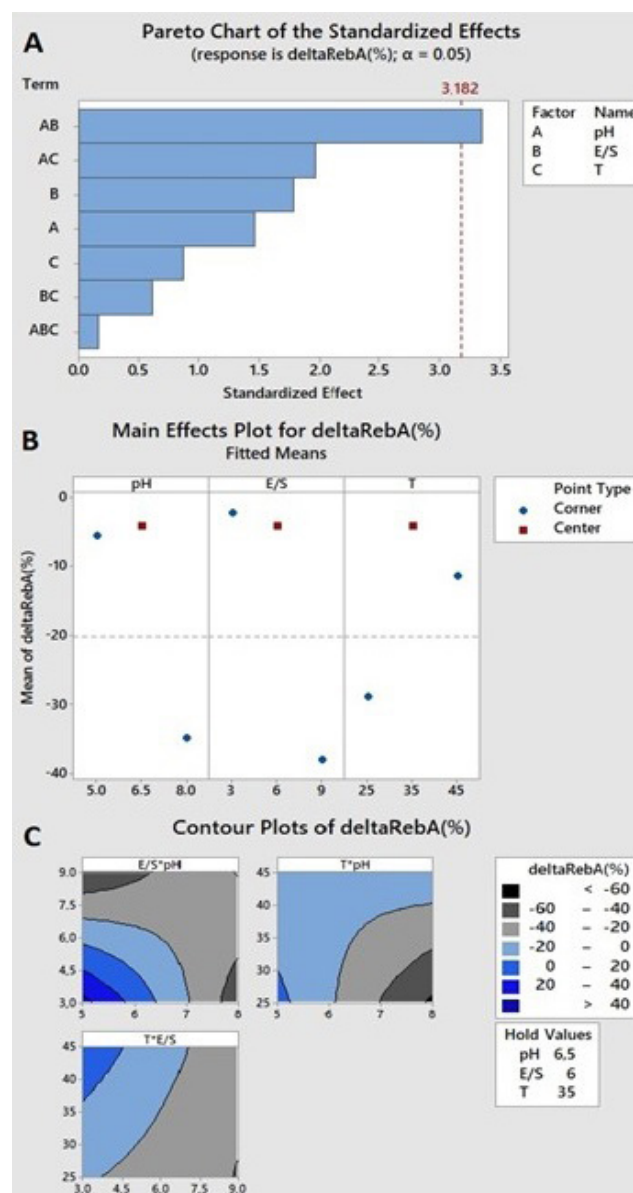


Fig. 9 Glycosyl donor: curdlan, A: Pareto Chart, B: Main Effects Plot, C: Contour Plot

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